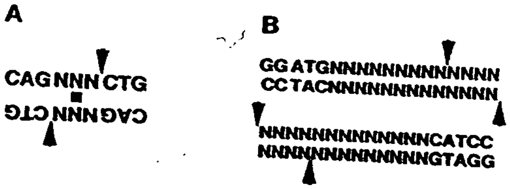
Two types of Hapaxomers

- Internal Palindromic Type II enzymes
  - e.g., *Sfi* I

    G G C C N N N N^N G G C C
    C C G G N^N N N N C C G G
- Outside Cutters (Type IIS)
  - e.g., *Sap* I

    G C T C T T C N^N N N
    C G A G A A G N N N N^

Fig 1


A
CAGNNNCTG
CAGNNNCTG
B
GGATGNNNNNNNNNNNNNN
CCTACNNNNNNNNNNNNNN
NNNNNNNNNNNNNNCATCC
NNNNNNNNNNNNNNGTAGG

Figs 2A-B

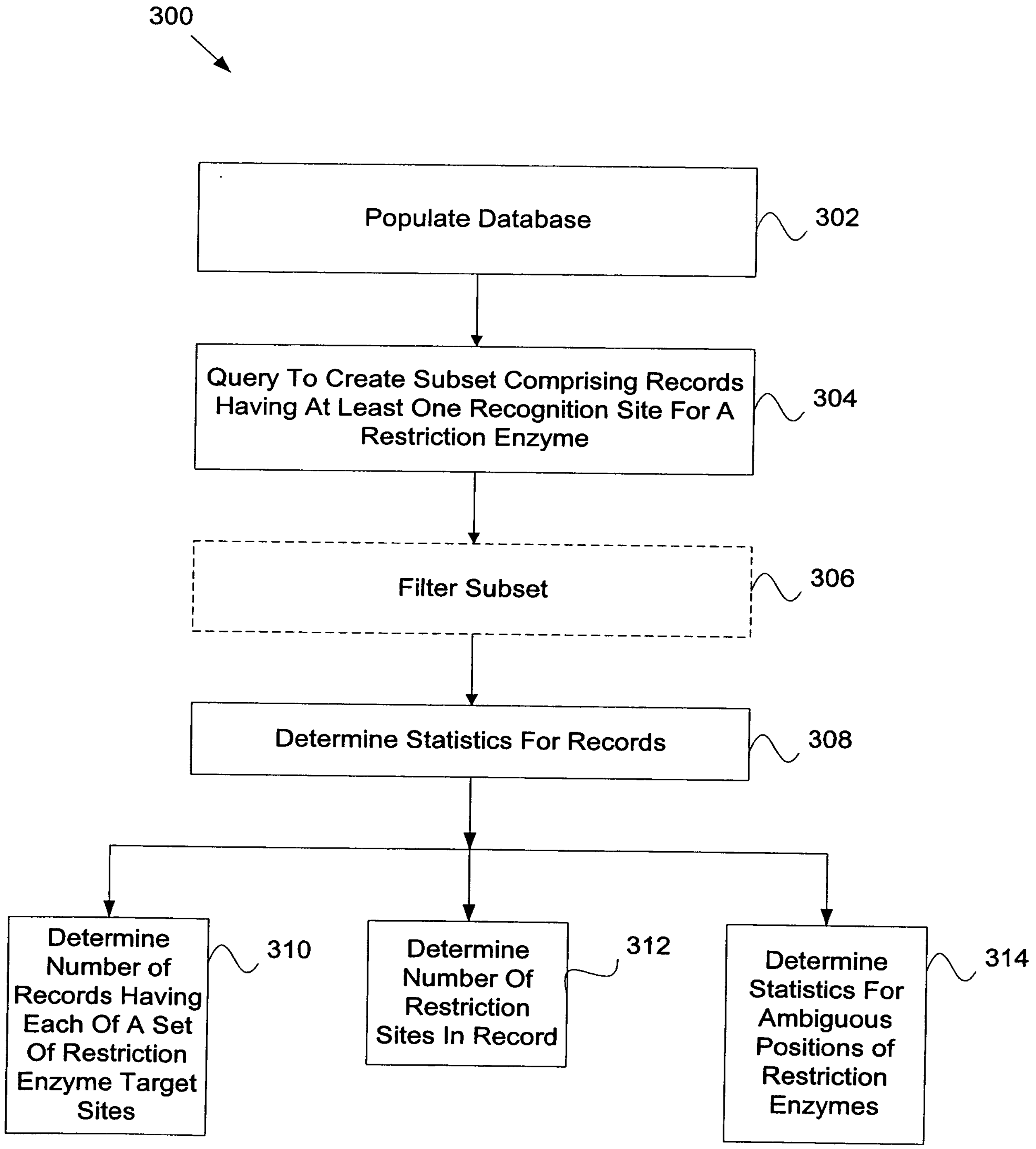

300
Populate Database
302
Query To Create Subset Comprising Records Having At Least One Recognition Site For A Restriction Enzyme
304
Filter Subset
306
Determine Statistics For Records
308
Determine Number of Records Having Each Of A Set Of Restriction Enzyme Target Sites
310
Determine Number Of Restriction Sites In Record
312
Determine Statistics For Ambiguous Positions of Restriction Enzymes
314

FIG. 3

	Sap I (0)	Sfi I (0)	Sfi I (0+1)	Sfi (0-2)	Sgf I/Pme I	Sap I (0)	Sap I (0+1)	Sap I (0-2)
Hs_Fna	56.72	85.93	96.72	98.91	97.45	56.72	84.67	94.82
Mgc	61.98	87.02	97.94	99.70	98.14	61.98	90.31	97.60
E coli	86.71	99.35	100.00	100.00	93.52	86.71	98.37	99.53
C elegans	65.93	99.46	99.97	100.00	98.55	65.93	90.38	97.19
S cerevisiae	80.02	99.15	99.98	100.00	97.17	80.02	96.68	99.29
Arabidopsis	70.83	99.56	100.00	100.00	97.57	70.83	93.37	98.63

Fig 4

7+ Cutters

Enzymes	Recognition Sequence	HsFna	MGC	Ec	Ce	Sc	At
AarI	CACCTGCNNNN^NNNN_	7142	5355				1138
AbeI	*CC^TCA_GC not available*	*7970*	*5836*	*141*	*90*	*374*	*1833*
AscI	GG^CGCG_CC	515	336	152	10	13	26
AsiSI	GCG_AT^CGC	108	62	207	39	29	178
BbvCI	*CC^TCA_GC*	*7970*	*5836*	*141*	*90*	*374*	*1833*
CciNI	GC^GGCC_GC	1444	823	19	33	31	97
CpoI	*CG^GWC_CG*	*1119*	*781*	*347*			
CspI	*CG^GWC_CG*	*1119*	*781*	*347*			
CspBI	GC^GGCC_GC not available	1444	823	19	33	31	97
Fs I	GG_CCGG^CC	1139	740	5	9	10	70
MabI	*A^CCWGG_T*						
MchAI	GC^GGCC_GC not available	1444	823	19	33	31	97
Mlu1106I	*RGGWCCY* not available						
NotI	GC^GGCC_GC	1444	823	19	33	31	97
PacI	TTA_AT^TAA	708	395	66	8	213	138
Pfl27I	*RG^GWC_CY* not available						
PpuMI	*RG^GWC_CY*						
PpuXI	*RG^GWC_CY*						
Psp5II	*RG^GWC_CY*						
PspPPI	*RG^GWC_CY*						
RsrII	*CG^GWC_CG*	*1119*	*781*	*347*			
Rsr2I	*CG^GWC_CG*	*1119*	*781*	*347*			
SanDI	*GG^GWC_CC*						
SapI	GCTCTTCN^NNN_	7260	4785	584	1296	1362	8870
SbfI	CC_TGCA^GG	2591	1802	60	13	66	251
SdaI	CC_TGCA^GG	2591	1802	60	13	66	251
SdiI	GGCCN_NNN^NGGCC not available	2214	1634	28	18	54	121
SexAI	*A^CCWGG_T*						
SfiI	GGCCN_NNN^NGGCC	2214	1634	28	18	54	121
SgfI	GCG_AT^CGC	108	62	207	39	29	178
SgrAI	CR^CCGG_YG						
Sse232I	CG^CCGG_CG not available	708	448	29	43	23	446
Ss 1825I	*GG^GWC_CC* not available						
Sse8387I	CC_TGCA^GG	2591	1802	60	13	66	251
Sse8647I	*AG^GWC_CT* not available						
VpaK32I	GCTCTTCN^NNN_ not available	7260	4785	584	1296	1362	8870

Six Cutters

Enzymes	Recognition Sequence	HsFna	MGC	Ec	Ce	Sc	At
NruI	TCG^CGA	830	607	1070	558	507	2422
SplI	C^GTAC_G	701	449	498	263	549	1705
SnaBI	TAC^GTA	1080	621	435	165	885	2164
PvuI	CG_AT^CG	842	512	1000	705	537	3078
MluI	A^CGCG_T	1049	1019	976	295	337	1824
BgI	GCCN_NNN^NGGC	8827	6868	1333	469	750	2576
Ear I	CTCTTCN^NNN_						
BsrGI	TGTACA	6683	4551	442	760	1583	5450
XmnI	GAANN^NNTTC	8401	5850	1167	1652	2911	12141
SalI	G^TCGA_C	1515	944	463	792	856	3616
BamHI	G^GATC_C	6426	4305	438	1047	1238	6782
KpnI	G_GTAC^C	4098	2755	442	305	1317	2992
EcoRI	G^AATT_C	6536	4132	470	1346	2466	8244
XhoI	C^TCGA_G	3651	2402	156	800	737	7092
EcoRV	GAT^ATC	3789	2435	1378	919	2289	8419
	AAA^TTT	7484	5167	1008	1049	3843	8078
DraI	TTT^AAA	8455	6243	967	494	3018	6778

Fig 5

. 7+ Blunt Cutters

Enzymes	Recognition S quence	HsFna	MGC	Ec	Ce	Sc	At
BstRZ246I	ATTT^AAAT	1204	648	55	38	379	317
BstSWI	ATTT^AAAT	1204	648	55	38	379	317
MspSWI	ATTT^AAAT	1204	648	55	38	379	317
MssI	GTTT^AAAC	297	173	71	9	152	490
PmeI	GTTT^AAAC	297	173	71	9	152	490
SmiI	ATTT^AAAT	1204	648	55	38	379	317
SwaI	ATTT^AAAT	1204	648	55	38	379	317
SrfI	GCCC^GGGC	1433	887	40	11	11	30

Hs_Fna	15740 entries
Mgc	12585 entries
Ec	4290 entries
Ce	3305 entries
Sc	6360 entries
At	27289 entries

Interrupted Palindromes

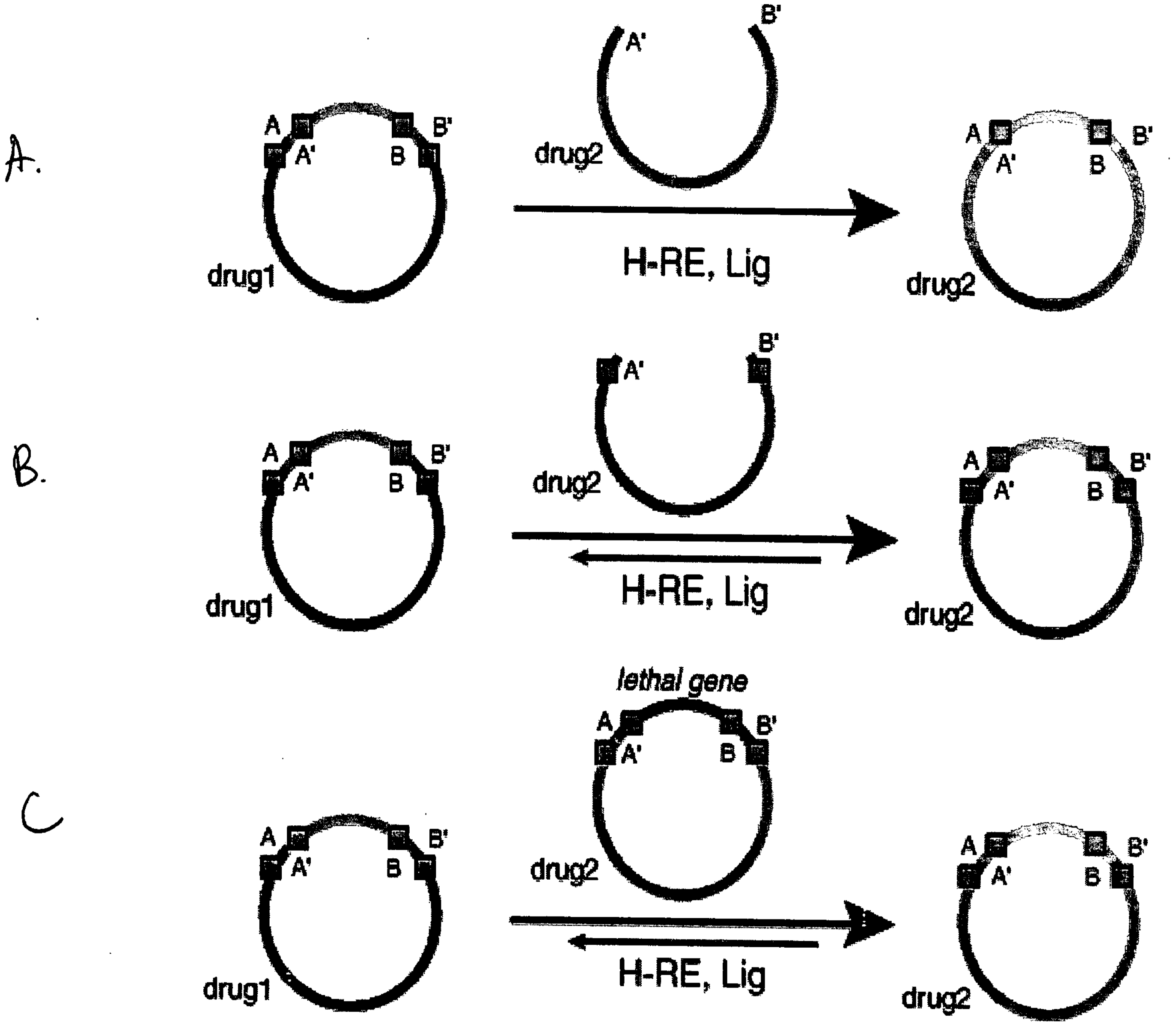


Fig 6

Sfi I

- How to make *Sfi* I “one way”
  - Methylases
  - *Bgl* I, not *Sfi* I sites, in Acceptor Vectors

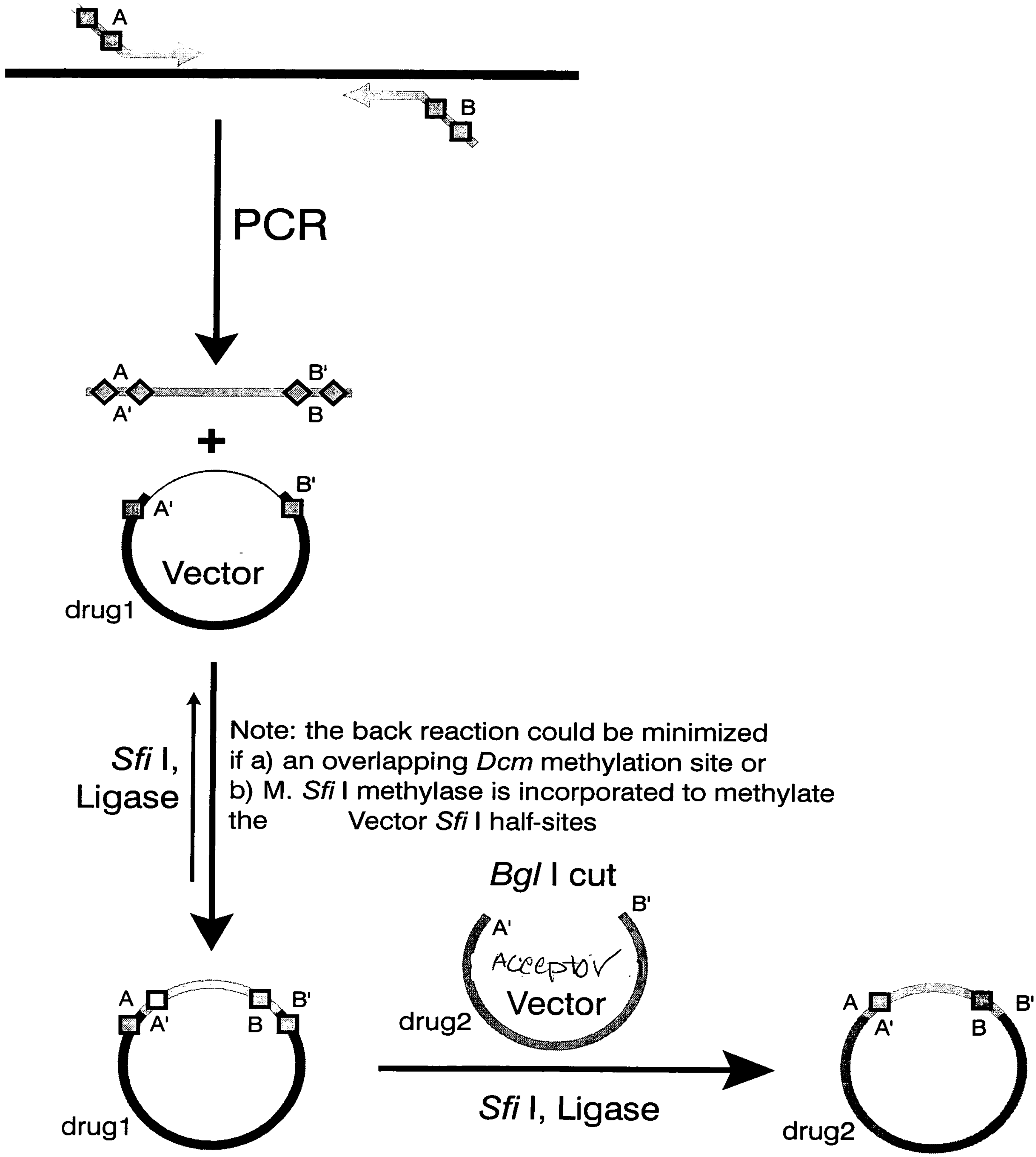
```
G G C C N N N N^N G G C C
C C G G N^N N N N C C G G

  G C C N N N N^N G G C
  C G G N^N N N N C C G
```

  - Lethal genes in stuffer fragments

Fig7

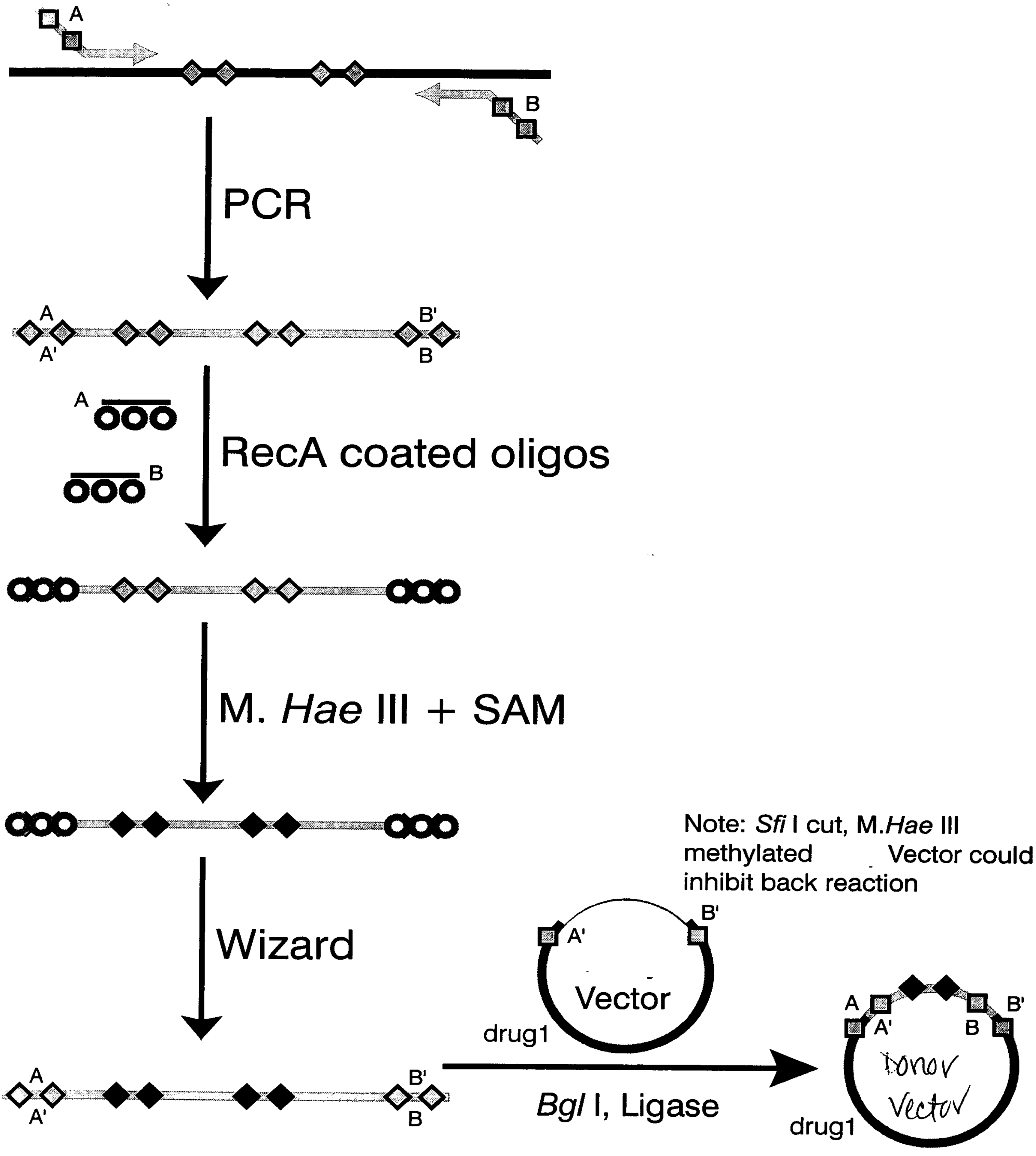
PCR Interrupted Palindrome Cloning Pathway



Note: the back reaction could be minimized if a) an overlapping *Dcm* methylation site or b) M. *Sfi* I methylase is incorporated to methylate the Vector *Sfi* I half-sites

Fig8

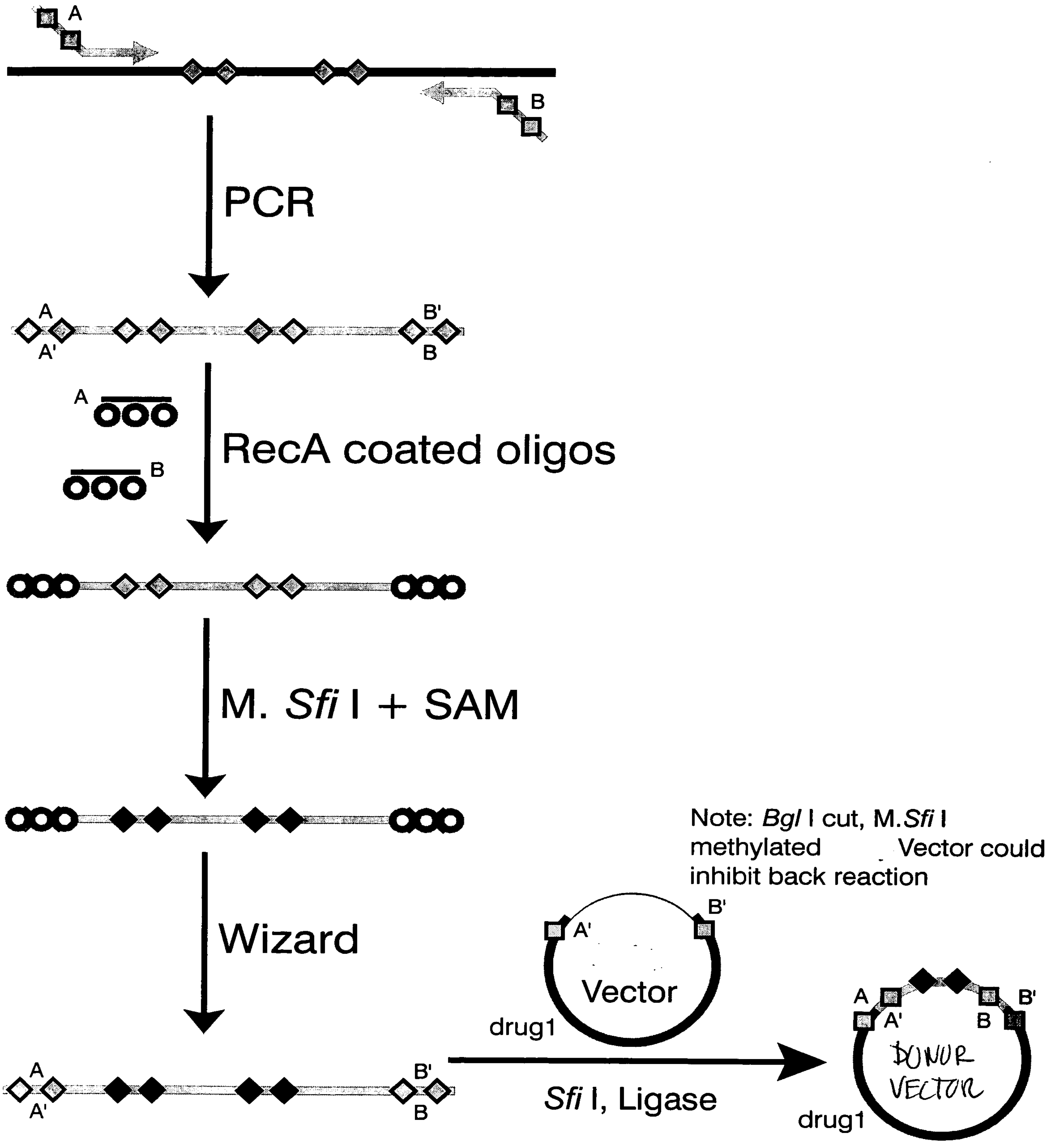
PCR Interrupted Palindrome Cloning Pathway 1A



Note: *Sfi* I cut, M.*Hae* III methylated Vector could inhibit back reaction

Fig 9A

PCR Interrupted Palindrome Cloning Pathway 2A



Note: *Bgl* I cut, M.*Sfi* I methylated Vector could inhibit back reaction

Fig 9B

PCR Interrupted Palindrome Cloning Pathway 1B

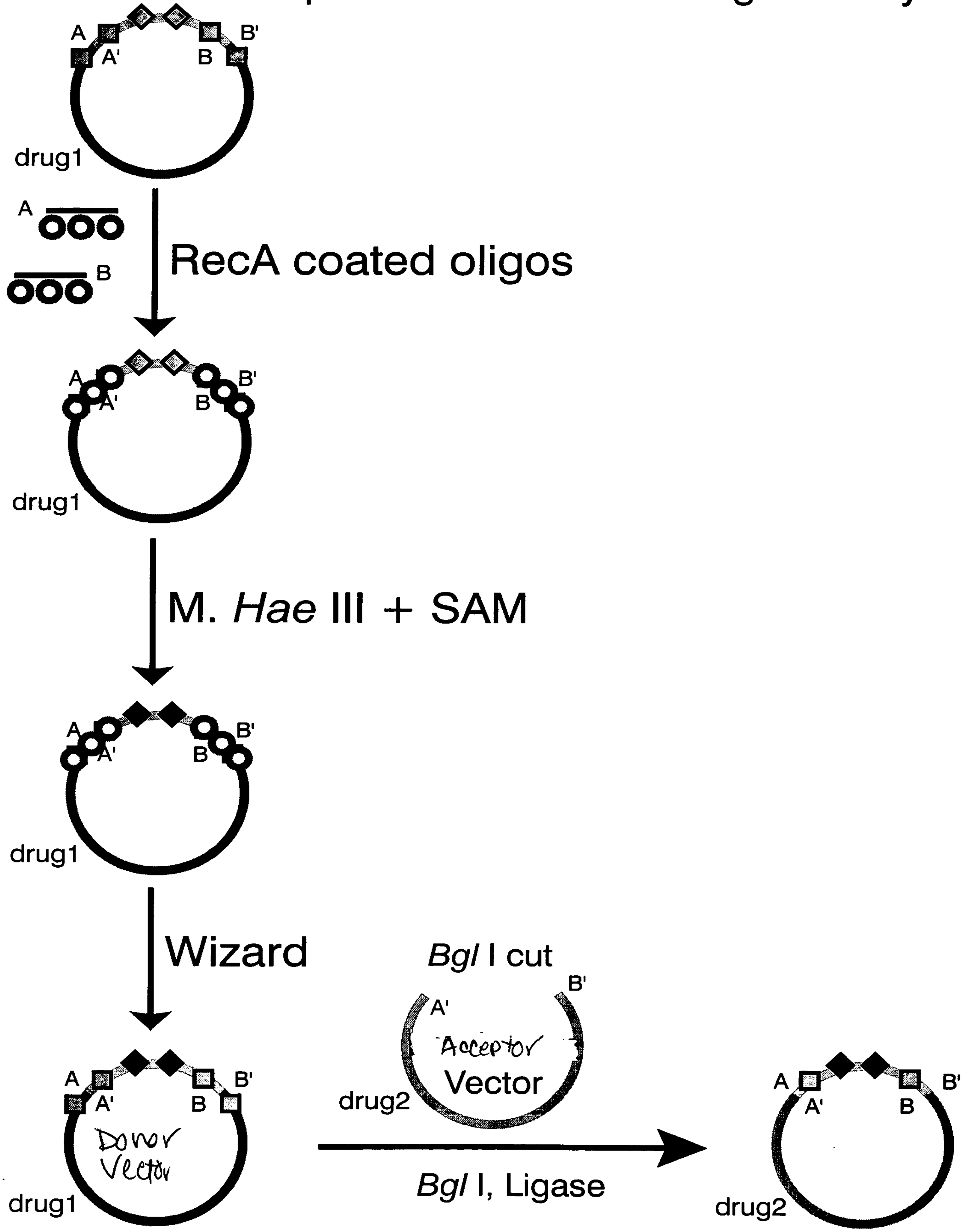


Fig10A

PCR Interrupted Palindrome Cloning Pathway 2B

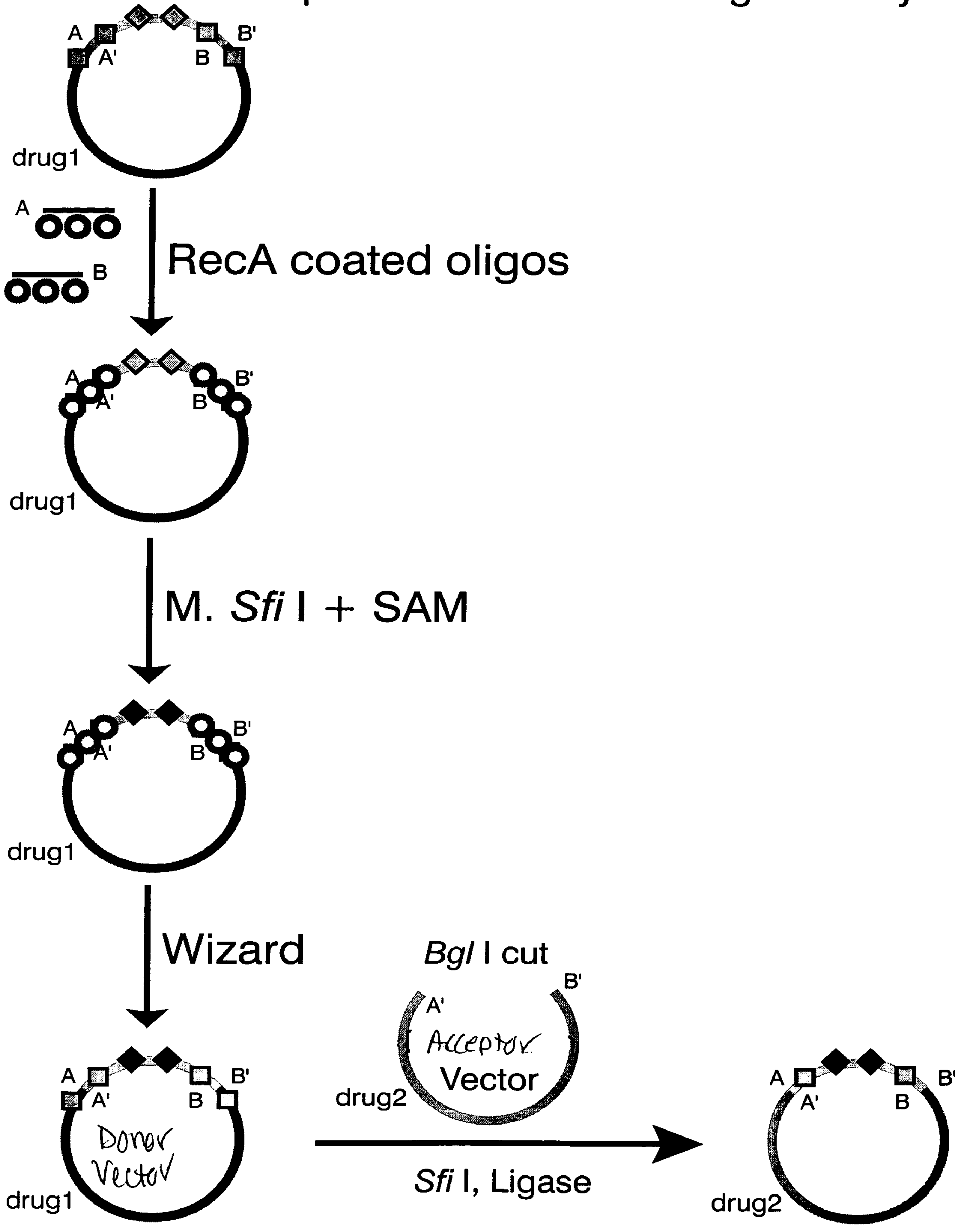


Fig
10B

REs that can make *Sfi* I one-way

3' 3b Overhang	Restriction Enzyme	Recognition Sequence
CNG	FmuI	G_GNC^C
CNG	PssI	RG_GNC^CY
CWG	Psp03I	G_GWC^C
GNC	BthCI	G_CNG^C
GSC	TauI	G_CSG^C
NNN	AlwNI	CAG_NNN^CTG
NNN	BglI	GCCN_NNN^NGGC
NNN	BsiYI	CCNN_NNN^NNGG
NNN	BstAPI	GCAN_NNN^NTGC
NNN	DraIII	CAC_NNN^GTG
NNN	MwoI	GCNN_NNN^NNGC
NNN	PflMI	CCAN_NNN^NTGG
NNN	RleAI	CCCACANNNNNNNNN_NNN^
NNN	SfiI	GGCCN_NNN^NGGCC

Fig 11

Outside Cutters (Type IIS)

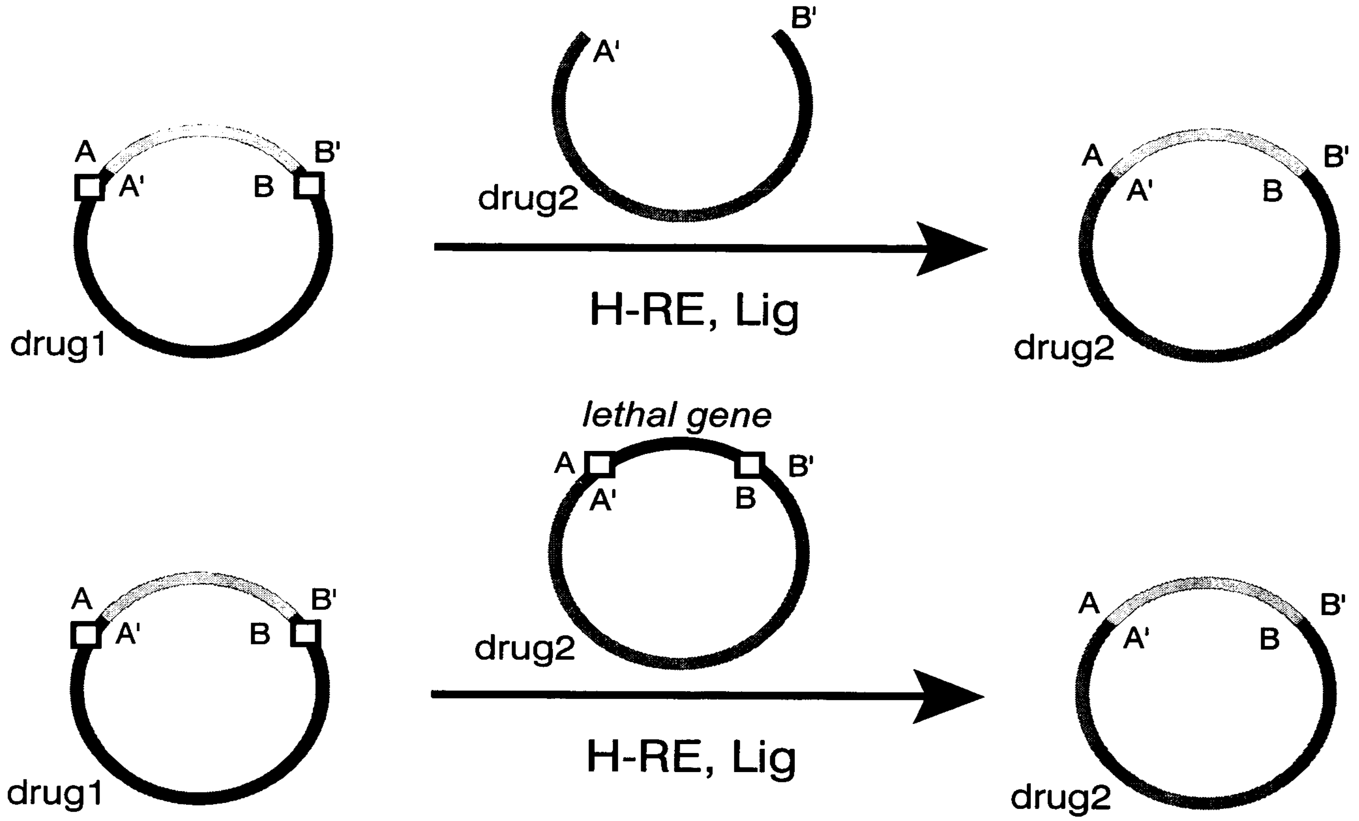


Fig 12

Sap I

- How to make *Sap* I "one way"
  - Methylases
  - Orientation of sites in vector backbone in Donor Vector and in Acceptor Vector
  - Lethal genes in stuffer fragments
  - *Ear* I, not *Sap* I sites, in Acceptor Vectors

```
G C T C T T C N^N N N
C G A G A A G N N N N^

  C T C T T C N^N N N
  G A G A A G N N N N^
```

- Key Advantage of *Sap* I
  - Only three bases per exchange site left in Acceptor Vector

Fig 13

Two Enzyme Approach

Sgf I – infrequent cutter of human cDNAs, two base 3’ overhang

```
G C G A T^C G C
C G C^T A G C G
```

Pme I – infrequent cutter, blunt end cutter

```
G T T T^A A A C
C A A A^T T T G
```

FIG 14A

Two Enzyme Approach

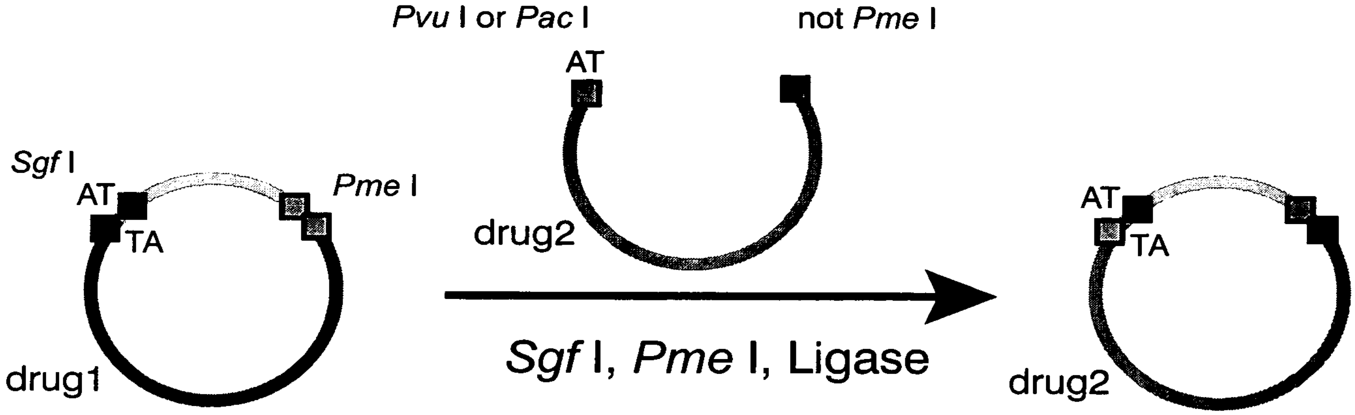
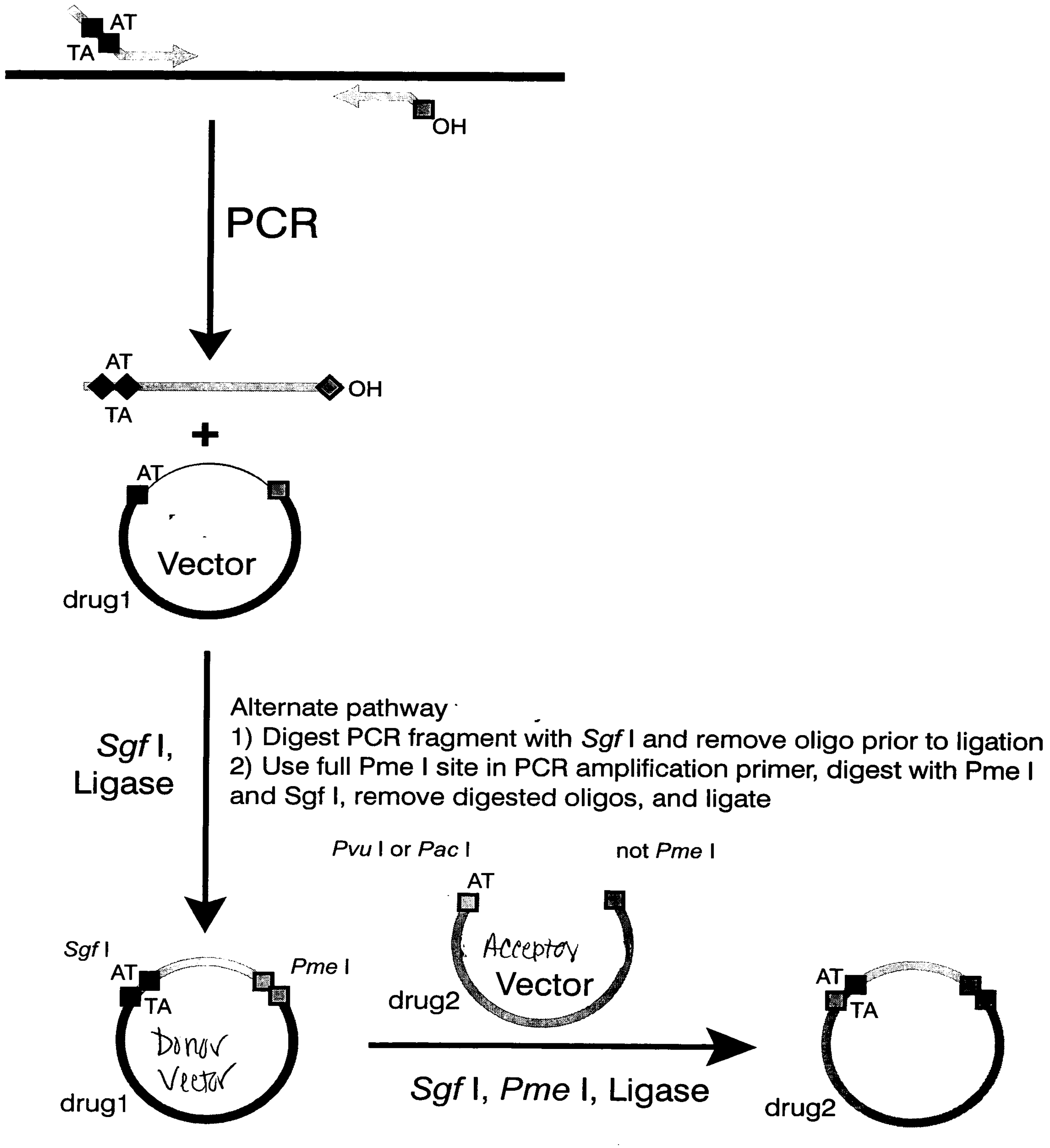


Fig 14B

Two Enzyme Cloning Pathway with PCR Entry



Alternate pathway
1) Digest PCR fragment with *Sgf* I and remove oligo prior to ligation
2) Use full Pme I site in PCR amplification primer, digest with Pme I and Sgf I, remove digested oligos, and ligate

FIG 15

N-terminal *Sgf* I site can allow N-terminal fusions OR *NO fusion*

NAAGGAGCGATCGCCATGg

--RBS-   Kozak--

VAAGGAGCGATCGCCATG

KEQGlyAlaIleAlaMet

Fig1h

C-terminal *Pme* I site allows termination (+1AA) or C-terminal fusions

NNNGTTTAAACN

XaaValTer

NNNGTTTATCN with *Eco*RV

XaaValTyr

NNNGTTTCCAN with *Bal*I, etc.

XaaValSer

Fig17

Coexpression Variation

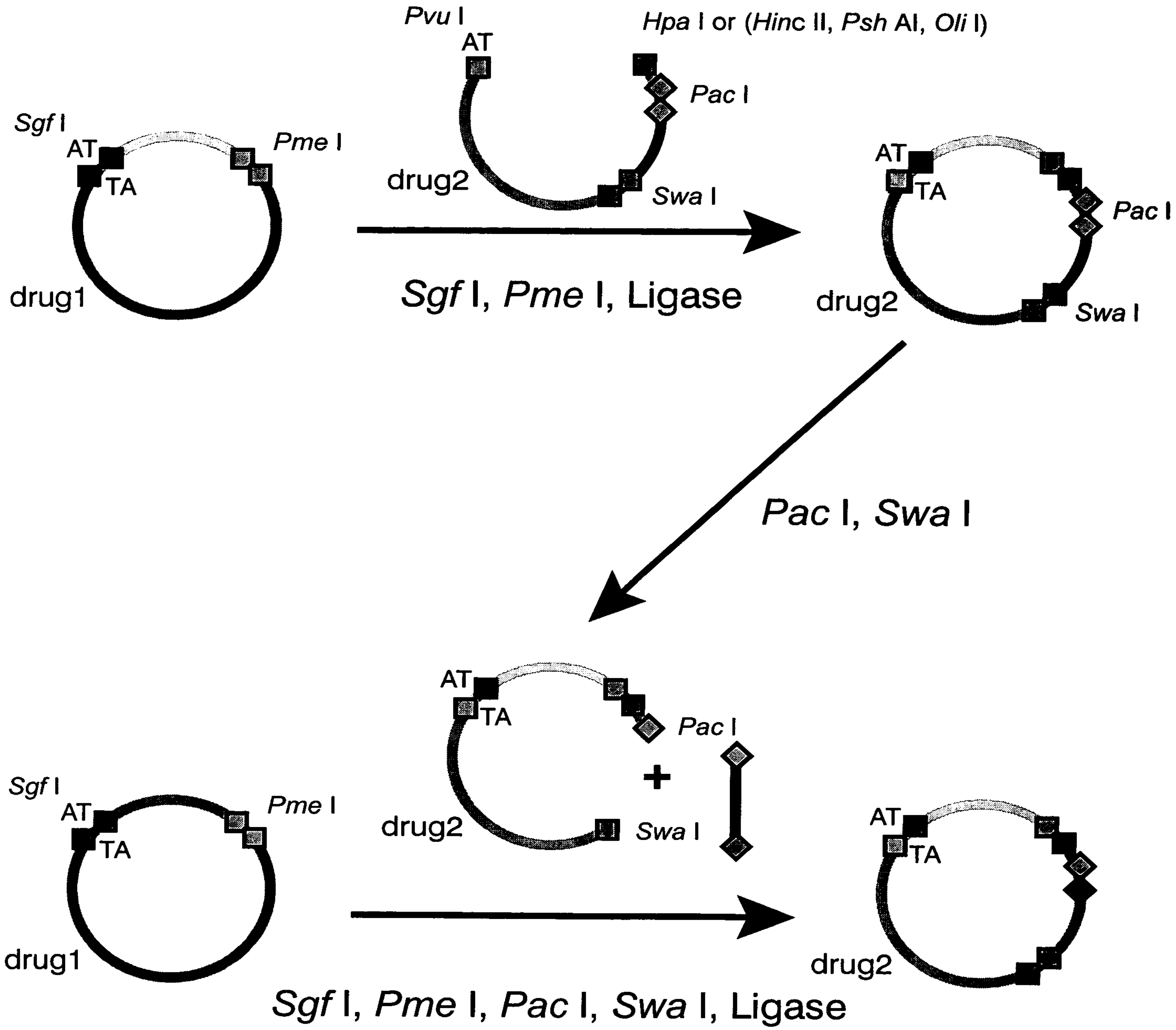


Fig18

N-terminal *Pac* I--*Sgf* I fusion site

NAAGGATTAATCGCCATGg

KEQGlyLeuIleAlaMet

C-terminal *Pme* I--*Swa* I fusion site

NNNGTTTAAATN

XaaValTer

Fig 19A

N-terminal *Pac* I--*Sgf* I fusion site

NAAGGATTAATCGCCATGg

--RBS- Kozak--

C-terminal *Pme* I--*Swa* I fusion site

NNNGTTTAAATN

XaaValTer

Fig 19B

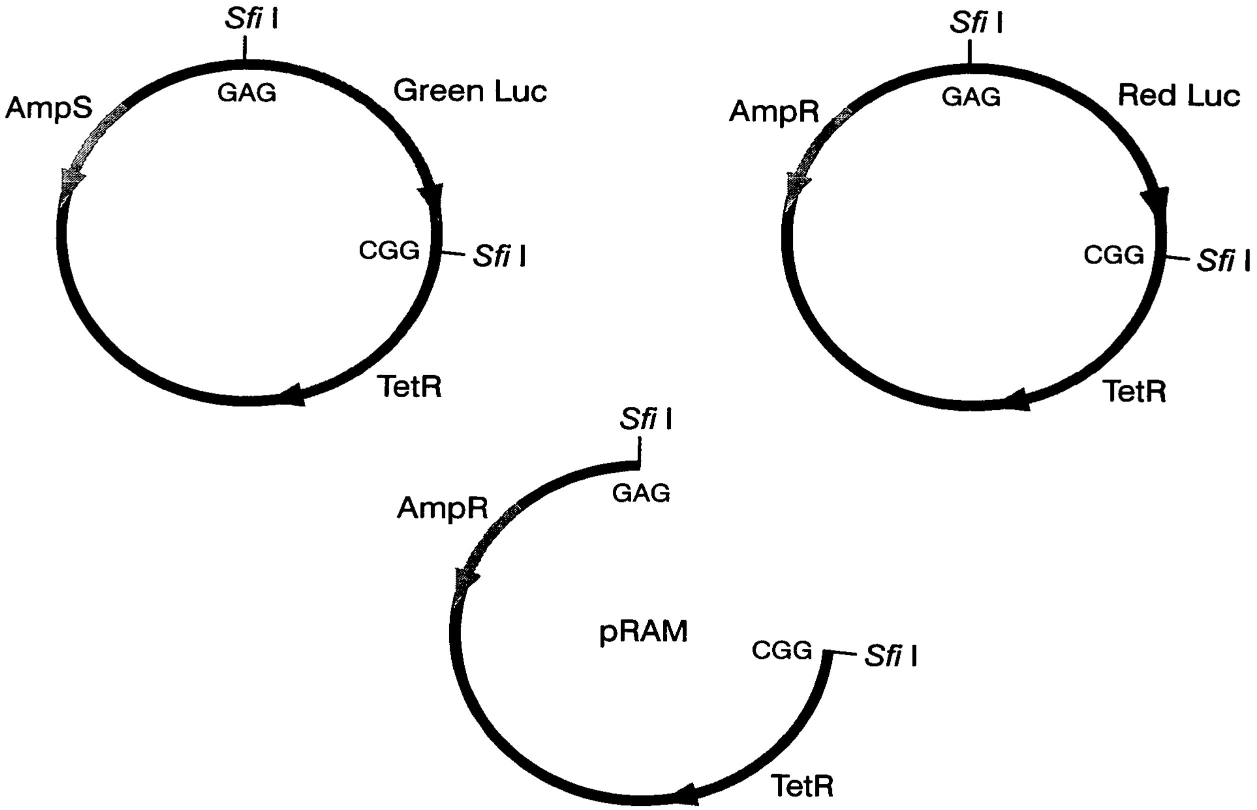

Sfi I
AmpS
GAG
Green Luc
CGG
Sfi I
TetR
Sfi I
AmpR
GAG
Red Luc
CGG
Sfi I
TetR
Sfi I
AmpR
GAG
pRAM
CGG
Sfi I
TetR

FIG20A

Sfi I cuts in ligase buffer, and cut ends religate with T4 DNA ligase

$i = N_0 M x 10^3$ ends/ml for non-identical cohesive termini
$j = j\lambda(MW\lambda/MW)^{3/2}$ ends/ml

i=total concentration of DNA termini
j=effective concentration of one end of a DNA molecule in the immediate neighborhood of the other end of the same molecule

Note: *Sfi* I cut at 50°C; ligase at 22°C

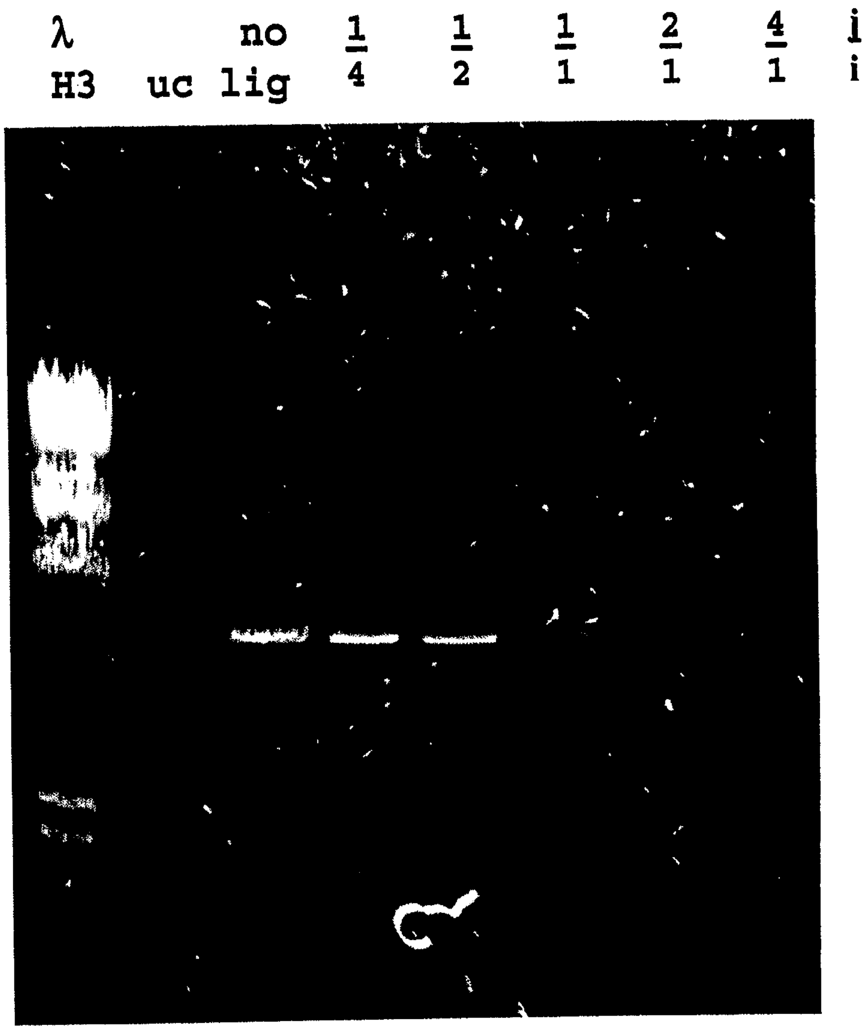


Fig 20B

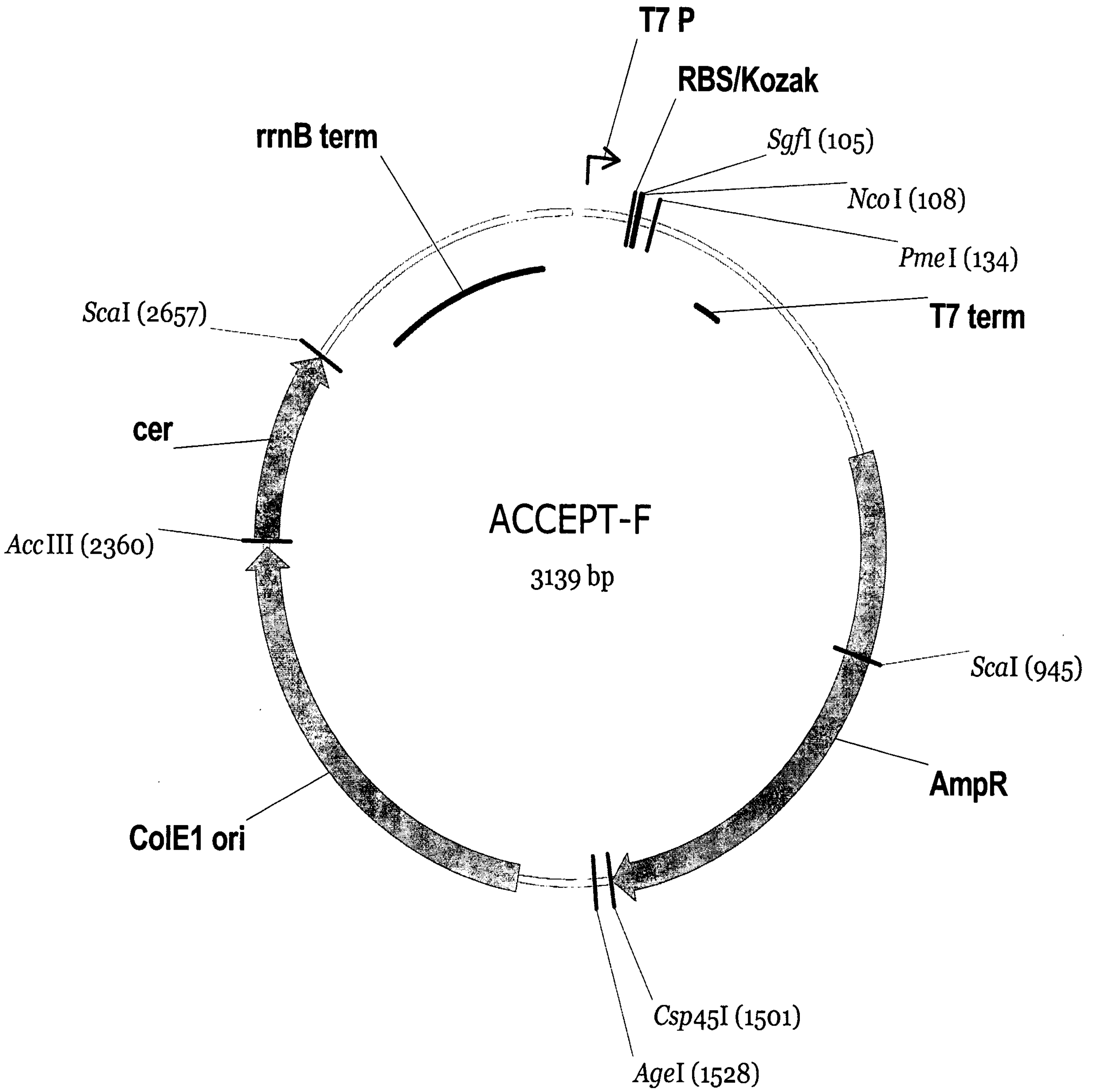
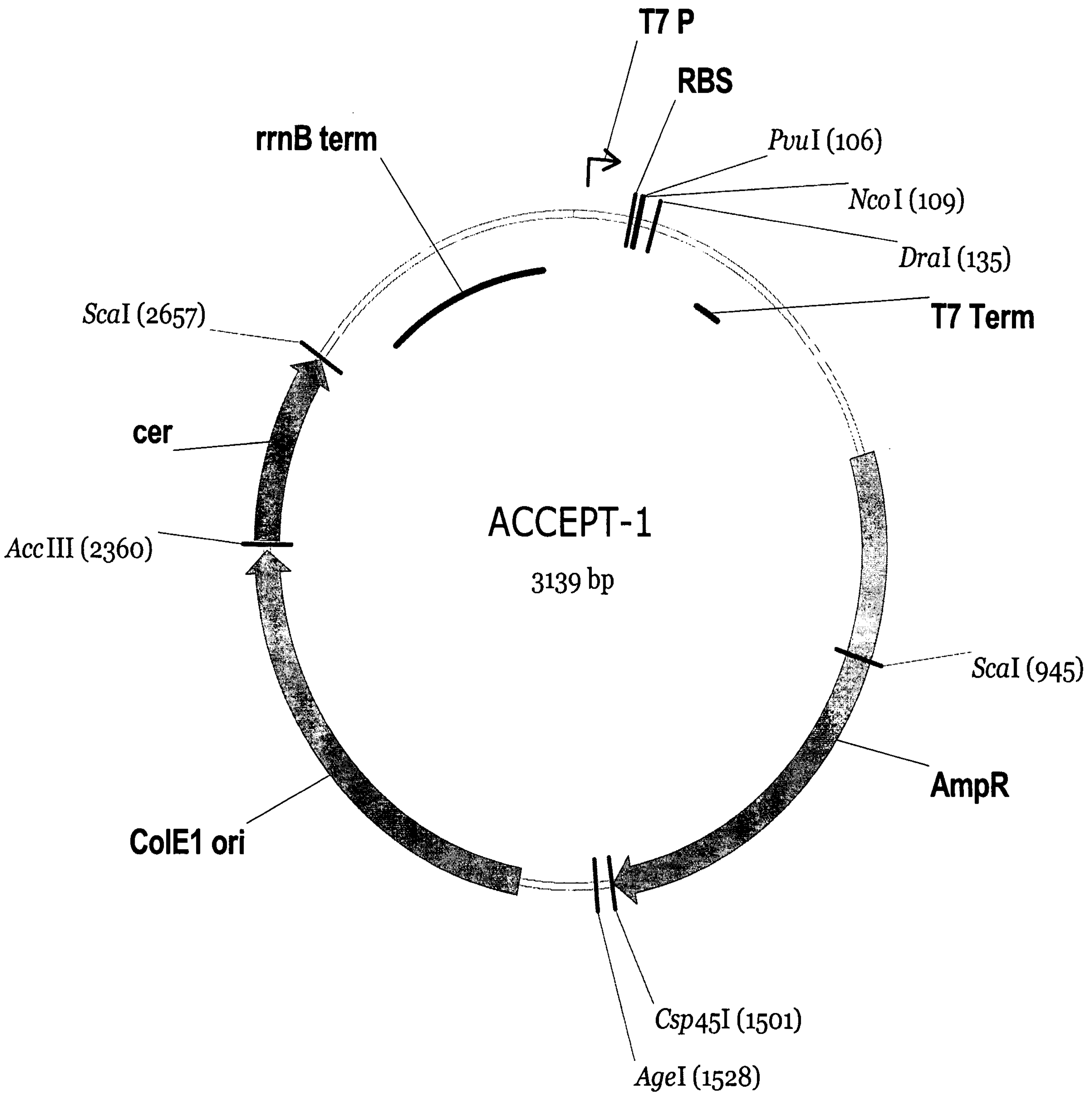
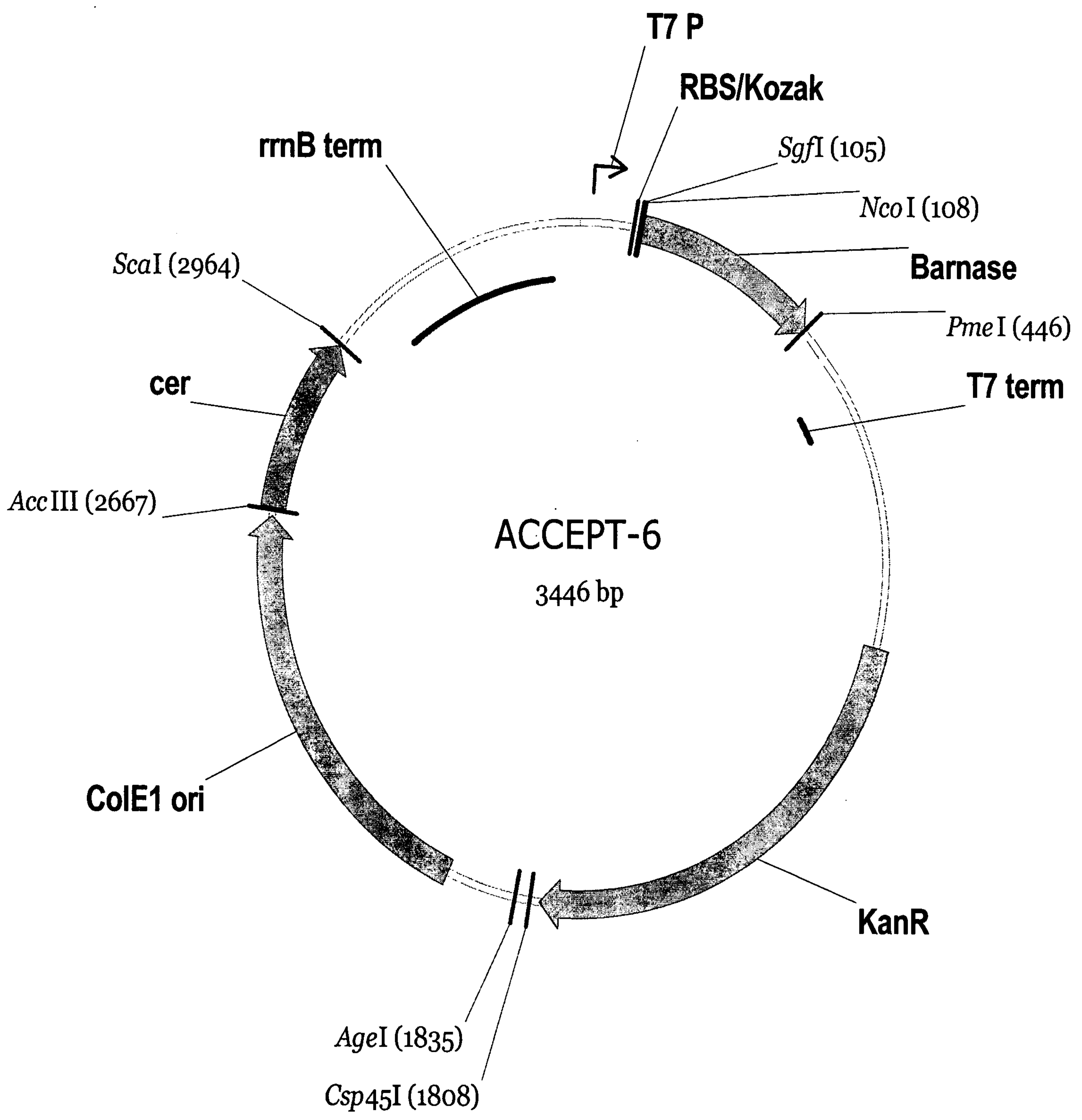
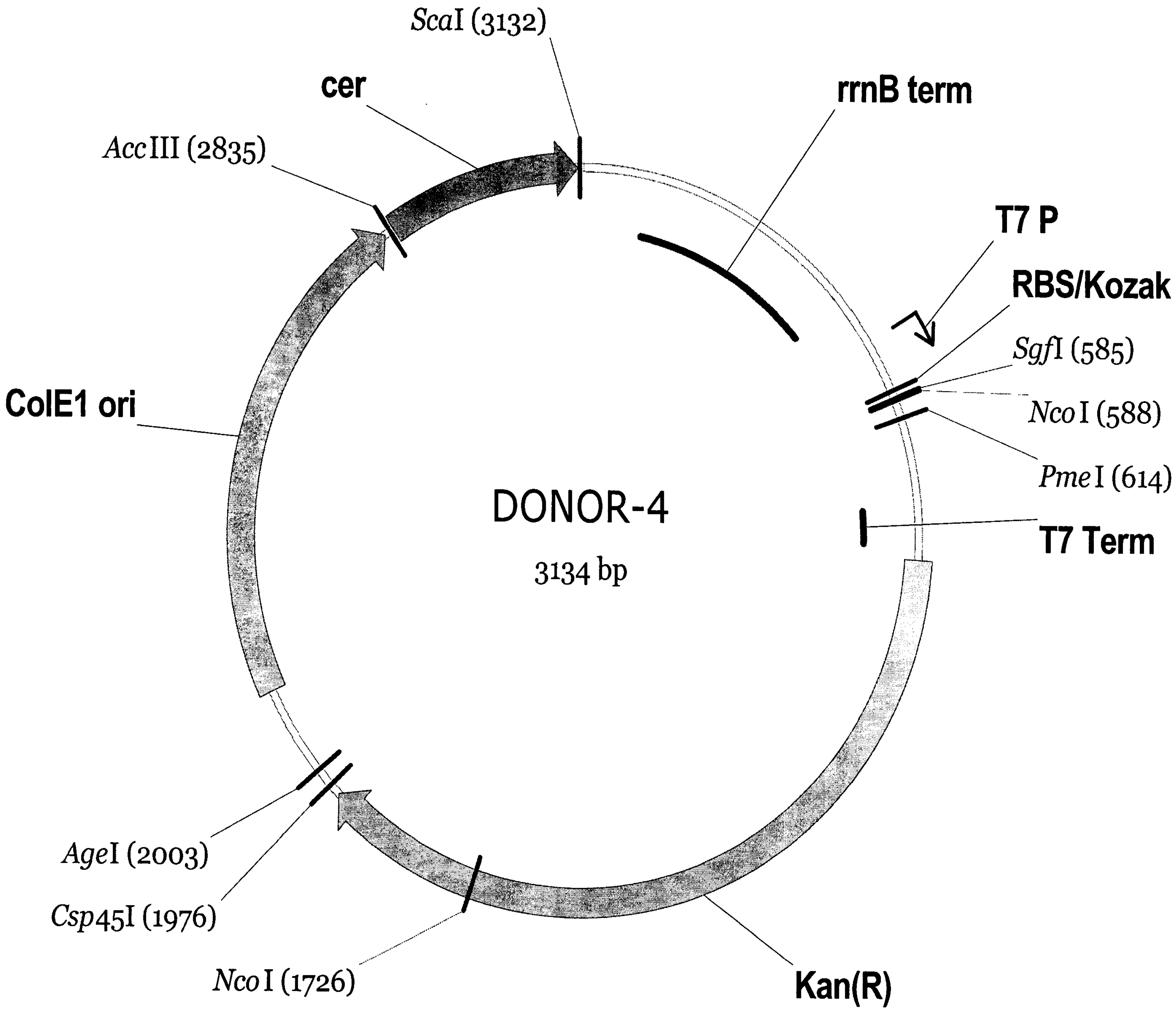
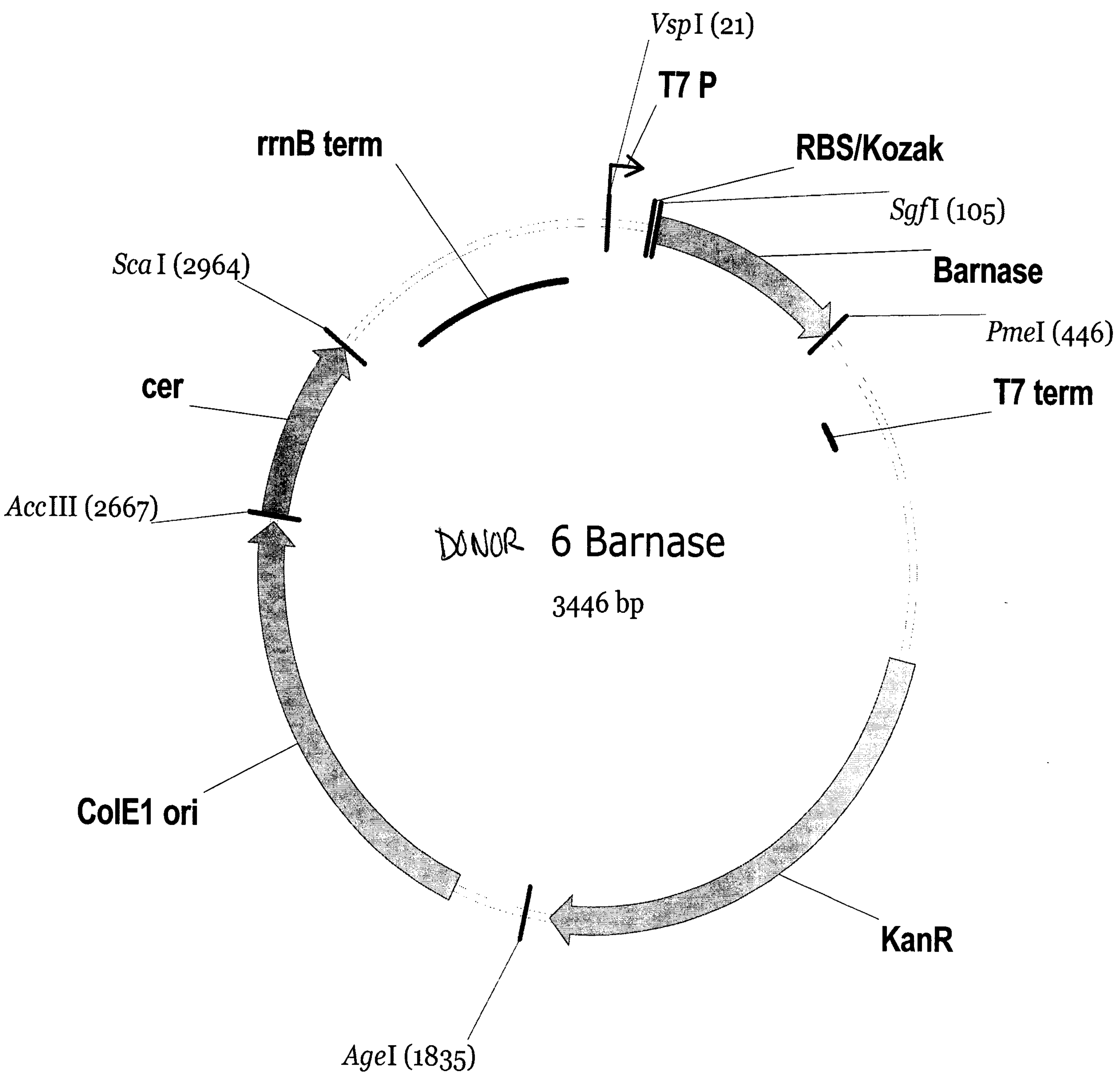

T7 P
RBS/Kozak
rrnB term
*Sgf*I (105)
*Nco*I (108)
*Pme*I (134)
T7 term
*Sca*I (2657)
cer
ACCEPT-F
3139 bp
*Acc*III (2360)
*Sca*I (945)
AmpR
ColE1 ori
*Csp*45I (1501)
*Age*I (1528)

Fig 21A-E


T7 P
RBS
*Pvu*I (106)
*Nco*I (109)
*Dra*I (135)
T7 Term
rrnB term
*Sca*I (2657)
cer
*Acc*III (2360)
ACCEPT-1
3139 bp
*Sca*I (945)
AmpR
ColE1 ori
*Csp*45I (1501)
*Age*I (1528)


T7 P
RBS/Kozak
rrnB term
*Sgf*I (105)
Nco I (108)
Barnase
*Sca*I (2964)
Pme I (446)
cer
T7 term
Acc III (2667)
ACCEPT-6
3446 bp
ColE1 ori
KanR
*Age*I (1835)
*Csp*45I (1808)


ScaI (3132)
cer
rrnB term
AccIII (2835)
T7 P
RBS/Kozak
SgfI (585)
NcoI (588)
PmeI (614)
ColE1 ori
DONOR-4
3134 bp
T7 Term
AgeI (2003)
Csp45I (1976)
NcoI (1726)
Kan(R)

Vsp I (21)
T7 P
RBS/Kozak
Sgf I (105)
Barnase
Pme I (446)
T7 term
rrnB term
Sca I (2964)
cer
Acc III (2667)
DONOR 6 Barnase
3446 bp
ColE1 ori
KanR
Age I (1835)

Luciferase Expression in 3 Hosts

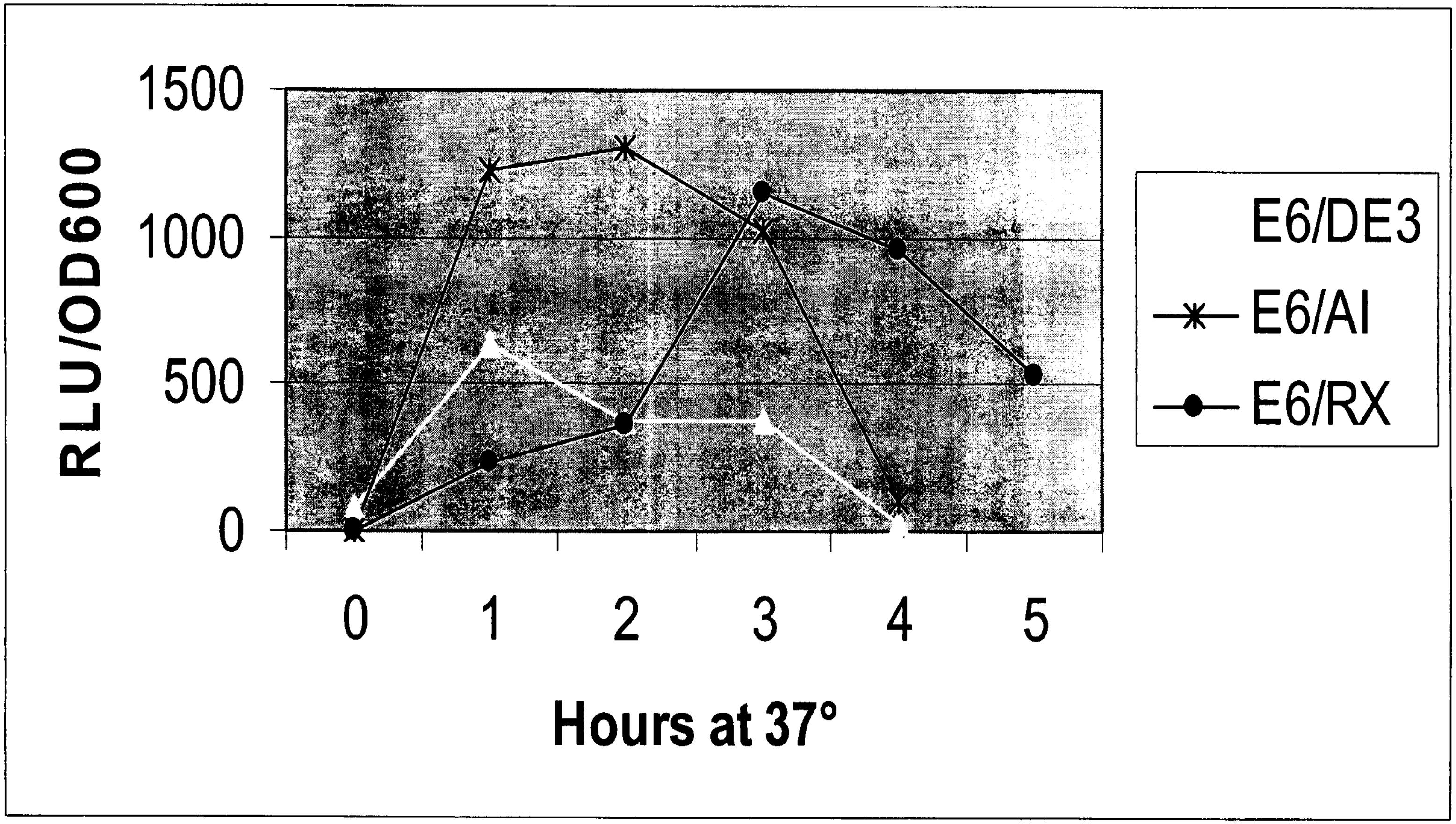
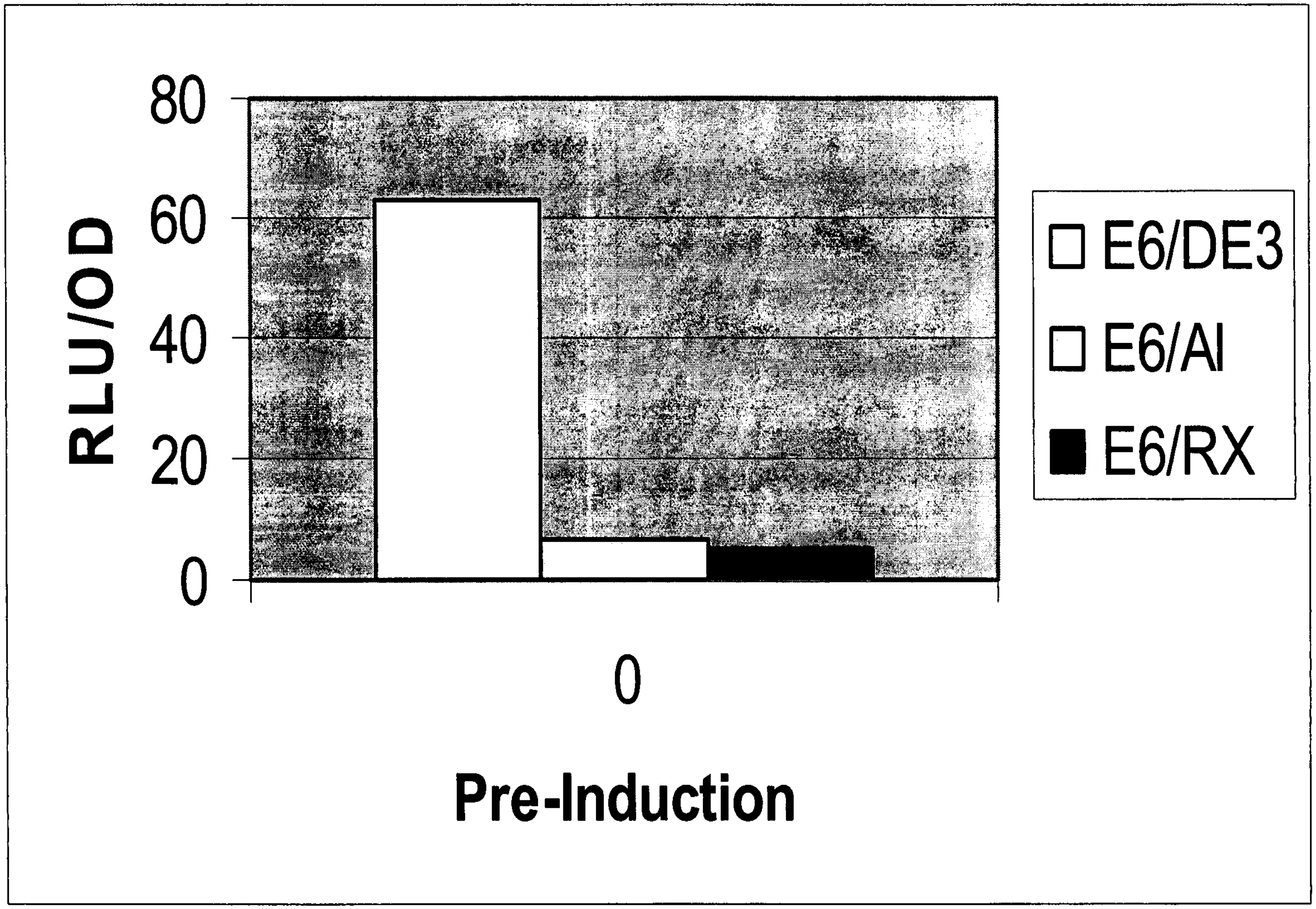


Fig 22A-C

Luciferase Expression in 3 Hosts at 25°C



Luciferase Expression in 3 Hosts at 25°C